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ISOLATION AND SCREENING OF POTENTIAL BACTERIOPHAGES FOR INHIBITION OF *Pseudomonas* spp. FROM SHRIMP'S SAMPLES

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ABSTRACT

Pseudomonas spp. are significant bacterial pathogens in farmed shrimp, causing substantial economic losses in aquaculture. This study investigated the potential of bacteriophages to control *Pseudomonas* spp. isolated from shrimp. Eight *Pseudomonas* spp. isolates were screened against 28 bacteriophage strains to determine their host range. Bacteriophage strains ΦXP4.1, ΦXP2.1, and ΦXP2.2 were selected for further analysis, including plaque assays and biofilm experiments, due to their ability to infect several *Pseudomonas* spp. isolates. All three phages effectively infected *Pseudomonas* sp. VCST7 and reduced bacterial density. Notably, ΦXP4.1 demonstrated the highest efficacy, reducing bacterial density by 11.08% and biofilm biomass by 66.3%. Transmission electron microscopy revealed that ΦXP4.1 belongs to the class *Caudoviricetes,* with an average head diameter of 61.301 nm and an average tail length of 135.7 nm. These findings suggest that bacteriophages, particularly ΦXP4.1, hold promise as biocontrol agents against *Pseudomonas* spp. in aquaculture.

Keywords: Bacterial host, bacteriophages, , double-layer agar, *Pseudomonas* spp., shrimp.

INTRODUCTION

*Pseudomonas*spp. are Gram-negative, aerobic, rod-shaped bacteria belonging to the family *Pseudomonadaceae*. These bacteria are typically 1-1.5 µm in length and 0.5-1 µm in width, and they can occur as single cells, in pairs, or occasionally in chains under microscopic observation. Motility is conferred by one or more polar flagella. *Pseudomonas* spp. are ubiquitous microorganisms widely distributed in diverse environments, including soil, water, air, and the surfaces of plants, animals, and humans (Duong & Thai, 2023; Lai Trinh Anh *et al*., 2024). In shrimp aquaculture, *Pseudomonas* spp. are associated with a range of diseases, including shell and soft tissue necrosis, intestinal infections, and systemic bacterial infections, which not only degrade shrimp quality but also diminish the commercial value of export shipments. The pathogenesis of *Pseudomonas* is complex and involves the production of various extracellular enzymes and toxins, such as pyocyanin and pyoverdine, which contribute to tissue damage and weaken the host's

immune defenses (Kassob & Hummadi, 2023).

The increasing prevalence of antibiotic-resistant *Pseudomonas* strains poses a significant threat to the sustainability of shrimp aquaculture. The overuse and misuse of antibiotics in aquaculture have contributed to the emergence and spread of resistance, rendering conventional treatments ineffective. For example, studies have reported high resistance rates in *Pseudomonas* spp. isolates against commonly used antibiotics such as ciprofloxacin, gentamicin, and imipenem (Tran *et al*., 2021). This resistance not only complicates disease management but also raises concerns about the potential transfer of resistance genes to other bacteria, including human pathogens.

In this context, bacteriophage therapy has emerged as a promising alternative strategy for controlling bacterial infections in aquaculture. Bacteriophages, viruses that specifically infect bacteria, offer several advantages over traditional antibiotics. They exhibit high host specificity, targeting particular bacterial strains while minimizing disruption to the shrimp's natural microbiota. This specificity reduces the risk of secondary infections and the selection of antibiotic-resistant bacteria (Kurt *et al*., 2025).

Bacterial biofilms, complex communities of bacteria embedded in a self-produced extracellular polymeric substance (EPS) matrix, further complicate the treatment of bacterial infections (Lawrence *et al*., 1991; Lochab *et al*., 2020). Biofilms provide bacteria with increased resistance to antibiotics and host immune defenses, making them particularly challenging to eradicate (Kunisch *et al*., 2024). *Pseudomonas* spp. are well-known biofilm formers, and biofilm formation on aquaculture equipment and within shrimp tissues can contribute to the persistence of infections (Kassob & Hummadi, 2023).

Numerous studies have demonstrated the potential of bacteriophages to control pathogenic bacteria, including *Pseudomonas* spp. (Low *et al*., 2020; Sharma *et al*., 2021; Duc *et al*., 2020). Bacteriophages have been shown to effectively lyse planktonic bacteria and degrade biofilms, offering a dual approach to combatting bacterial infections. For instance, studies have reported the successful use of bacteriophages to control *P. aeruginosa* in various settings, highlighting their potential for application in aquaculture (Kwiatek *et al*., 2017).

The study aimed to isolate and identify phage strains capable of controlling *Pseudomonas* spp. from shrimp. First, we isolated *Pseudomonas* spp. strains and bacteriophage strains from shrimp. Next, we evaluated the host range and infectivity of the isolated phage strains through turbidity measurements and spread plate counting. Additionally, we tested the ability of phage strains to degrade biofilms of *Pseudomonas* sp.. Finally, we characterized the morphology of the lysis plaques and promising phage strains. The findings of this study will contribute to exploring the potential of phages as an alternative therapy for the treatment of pathogenic bacteria in aquaculture, thereby contributing to the development of safe and environmentally friendly biological therapies.

MATERIALS AND METHODS

Bacterial isolation

Eight *Pseudomonas* spp. isolates were obtained from four shrimp samples. Shrimp

tissue was homogenized, mixed with 15 mL of distilled water, and then serially diluted. A 100 µL aliquot of the appropriate dilution was spread onto King's B agar plates. King's B agar was prepared according to the standard formulation: 20 g/L Proteose Peptone No. 3, 1.5 g/L $MgSO_4.7H_2O$, 1.5 g/L K_2HPO_4, and 15 g/L agar, pH 7.2. The plates were incubated at 30°C for 24-48 hours. Colonies exhibiting circular, smooth, and flat morphology, resembling a "fried egg" with serrated edges, a central core, and producing a blue-green pigment (indicative of pyocyanin production), were selected for further analysis (Mansoor & Ehteshamul-Haque, 2007).

Bacteriophage isolation and purification

To isolate bacteriophages, *Pseudomonas* spp. bacterial suspension was prepared in TSB (Tryptic Soy Broth) and incubated at 30°C until mid-log phase. The bacterial suspension was then treated with 1% (v/v) chloroform, mixed gently, and incubated at room temperature for 15 minutes to lyse the bacterial cells and release bacteriophages. The mixture was centrifuged at 12,000 rpm for 10 minutes to pellet cell debris, and the supernatant containing the crude bacteriophage lysate was collected. The presence of bacteriophage in the lysate was initially confirmed using the drop assay (Kropinski *et al*., 2009). Bacteriophages were purified by performing serial dilutions of the lysate and using the double-layer agar (plaque assay) method, as described by Kropinski*et al*. (2009), to obtain single, isolated plaques.

Bacteriophages host range

The host range of the bacteriophage strains was determined using the drop assay method. Briefly, a bacterial lawn of each *Pseudomonas* spp. isolate was prepared on King's B agar plates. A 10 µL drop of each bacteriophage suspension was spotted onto the bacterial lawn. The plates were incubated at 30°C for 24 hours, and the formation of clear plaques was observed and recorded (Kropinski *et al*., 2009; Sada & Tessema, 2024).

Investigation of phage infectivity through colony density

The effect of bacteriophages on bacterial viability was assessed using the spread plate method: A 100 µL aliquot of the *Pseudomonas* sp. VCST7 suspension (10^7 CFU/mL) was mixed with 100 µL of each bacteriophage solution in an Eppendorf tube at a multiplicity of infection (MOI) of 1. The mixture was incubated at 30°C for 30 minutes to allow for phage adsorption. Following incubation, the mixture was spread onto King's B agar plates and incubated at 30°Cfor 24 hours. Colony density was determined by manually counting colonies on the plates (Kropinski *et al*., 2009). The colony density is calculated based on the number of colonies present on the plate using the following formula (Ferdous *et al*., 2024):

$$\text{A (CFU/mL)} = \frac{\text{No. of } colonies\ per\ plate\ x\ Total\ dilution\ factor}{Volume\ of\ culture\ per\ plate\ in\ mL}$$

Biofilm formation assay

The ability of bacteriophages to degrade *Pseudomonas* spp. biofilms was assessed using a modified crystal violet staining assay, adapted from Stepanović *et al*. (2000). *Pseudomonas* sp. VCST7 was cultured in TSB at 30°C for 24 and 48 hours to allow for biofilm formation. Briefly, 200 µL of the bacterial suspension (10^7 CFU/mL) was

added to each well of sterile 96-well polystyrene microtiter plates. The plates were incubated statically at 30°C for 24 or 48 hours. After biofilm formation, the planktonic cells were carefully removed, and the wells were washed three times with 200 µL of sterile phosphate-buffered saline (PBS, pH 7.4) to remove non-adherent bacteria. Subsequently, 200 µL of the bacteriophage solution (10^8 PFU/mL) was added to each well, and the plates were incubated at 30°C for 4 hours. Control wells received 200 µL of sterile TSB. Following phage treatment, the solutions were removed, and the wells were washed three times with 200 µL of sterile PBS. The remaining biofilm was fixed with 200 µL of methanol for 15 minutes, after which the methanol was removed, and the plates were air-dried. The biofilm was stained with 200 µL of 0.1% (w/v) crystal violet solution for 15 minutes, followed by three washes with 200 µL of distilled water. After air-drying, the crystal violet bound to the biofilm was dissolved in 200 µL of 33% (v/v) glacial acetic acid, and the absorbance was measured at 595 nm using a microplate reader (Thermo Fisher Scientific).

Transmission electron microscopy

The purified bacteriophages (ΦXP4.1) at a concentration of 10^9 PFU/mL were absorbed onto carbon-coated copper grids for 5 minutes. Excess liquid was wicked away with filter paper, and the grids were negatively stained with 2% (w/v) uranyl acetate for 1 minute. The grids were air-dried and examined using a transmission electron microscope (TEM, JEOL JEM-1400) operated at the Kyoto Institute of Technology (KIT), Japan.

Statistical analysis

Statistical analysis was performed using MINITAB 16 software. Data are presented as the mean ± standard deviation from at least three independent experiments. Analysis of variance (ANOVA) was used to determine significant differences between treatment groups. When ANOVA indicated a significant difference, Tukey's HSD post-hoc test was used for pairwise comparisons. The assumptions of ANOVA (normality and homogeneity of variance) were assessed using the Shapiro-Wilk test and Levene's test, respectively. A p-value of < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Isolation and characterization of bacteria strains

Based on the isolation from shrimp samples, one bacterial strain, designated VCST7, exhibited morphological characteristics consistent with *Pseudomonas* spp. The VCST7 colonies formed on King's B agar after 48 hours were circular, smooth, and flat, with a distinct central core and a blue-green pigmentation (Figure 1A). Under a stereomicroscope, the colonies displayed a "fried egg" appearance with serrated edges (Figure 1B).

Genomic DNA was extracted from the VCST7 strain, and the 16S rRNA gene was amplified and sequenced to confirm its identity. The 16S rRNA gene sequence of VCST7 showed 100% similarity to *Pseudomonas* spp., confirming the bacterial isolate as belonging to the *Pseudomonas* genus. A neighbor-joining (NJ) phylogenetic tree was constructed to illustrate the evolutionary relationship of VCST7 with other *Pseudomonas* strains (Figure 2).

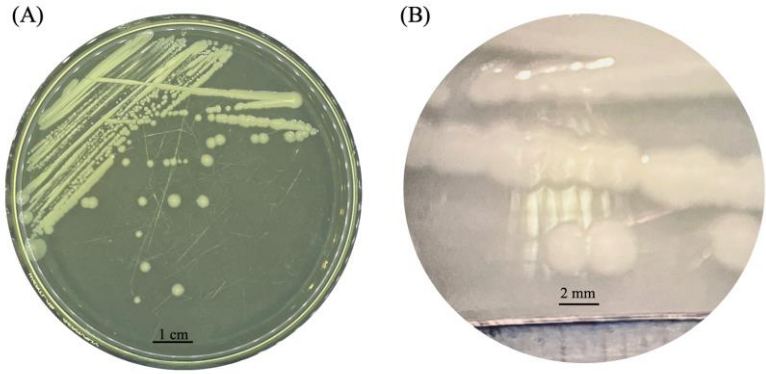

Figure 1. VCST7 bacterial colony on King's B agar after 48 hours. (A) Colonies on the agar plate, (B) Colonies under a stereomicroscope.

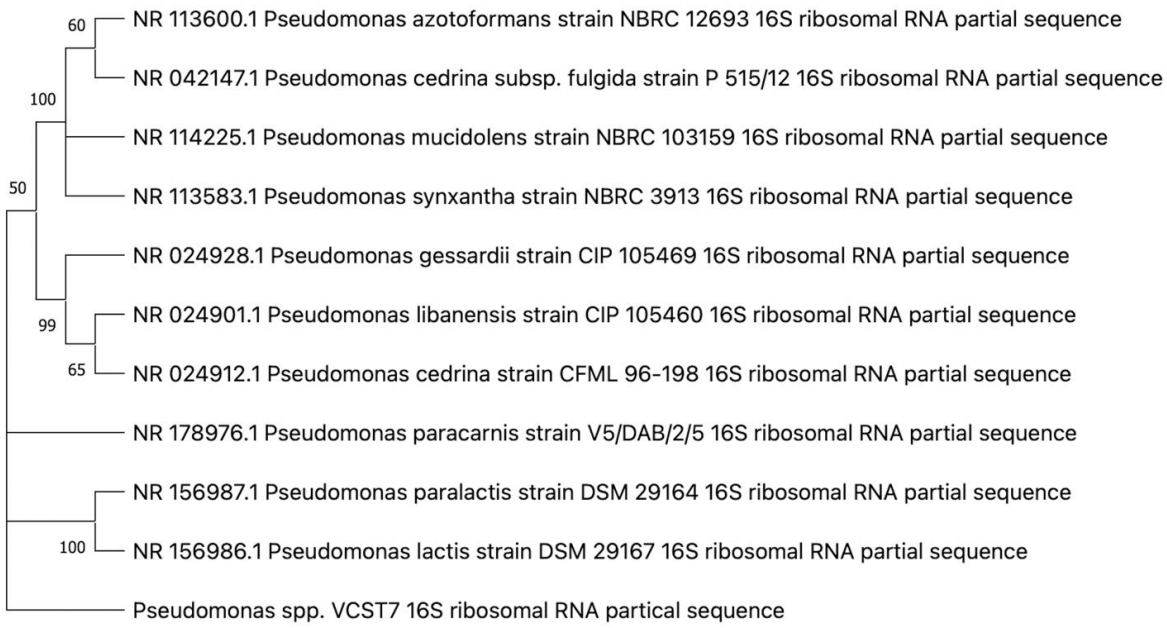

Figure 2. Phylogenetic trees of VCST7 based on the 16S rRNA sequences. Numbers at nodes represent bootstrap support values (1000 replicates).

Host specificity

A total of 28 bacteriophage strains (22 from the Advanced Virology Laboratory, Can Tho University, and 6 newly isolated in this study) were screened for their ability to infect eight *Pseudomonas* spp. isolates (Table 1). The host range assay revealed that bacteriophage strains ΦXP2 and ΦXP4 exhibited a relatively broad host range, successfully infecting 3 out of the 8 bacterial strains, particularly VCST4, VCST6, and VCST7 isolated from shrimp meat samples. Notably, the VCST7 strain demonstrated high susceptibility to the majority of the bacteriophages, with 26 out of 28 phages capable of forming clear plaque on this strain.

These results are consistent with previous studies demonstrating that some lytic bacteriophages can exhibit a broad host range within *Pseudomonas* spp. For example, de Melo *et al*. (2019) reported that bacteriophage ΦBrSP1 was capable of lysing 19 out of 37 *P. aeruginosa* strains. Similarly, Rombouts *et al*. (2016) highlighted the potential of lytic phages with broad host ranges for controlling *Pseudomonas* spp. infections. Based on these findings, bacteriophage strains ΦXP2 and ΦXP4 were selected for further purification and in-depth analysis of their effects on the *Pseudomonas* sp. VCST7 strain.

Table 1. Host range of 28 bacteriophage strains on 8 bacterial strains.

No.	Phages	Sources	Bacterial strains								
			VCS T1	VCS T2	VCS T3	VCS T4	VCS T5	VCS T6	VCS T7	VCS T8	No. of phages infecting bacteria
1	VP1	Shrimp	-	-	-	-	-	-	-	-	0
2	VP2	Shrimp	-	-	-	-	-	-	+	-	1
3	VP3	Shrimp	-	-	-	-	-	-	+	-	1
4	VP4	Shrimp	-	-	-	-	-	-	+	-	1
5	VP5	Shrimp	-	-	-	-	-	-	+	-	1
6	VP6	Shrimp	-	-	-	-	-	-	+	-	1
7	9MB	Pig intestine	-	-	-	-	-	-	+	-	1
8	10T	Pig intestine	-	-	-	-	-	-	+	-	1
9	9T	Pig intestine	-	-	-	-	-	-	-	-	0
10	1209	Shrimp water waste	-	-	-	-	-	-	+	-	1
11	233	Shrimp water waste	-	-	-	-	-	-	+	-	1
12	5A	Shrimp water waste	-	-	-	-	-	-	+	-	1
13	XP1	Shrimp	-	-	-	-	-	-	+	-	1
14	XP2	Shrimp water waste	-	-	-	+	-	+	+	-	3
15	XP3	Shrimp	-	-	-	-	-	-	+	-	1
16	XP4	Shrimp water waste	-	-	-	+	-	+	+	-	3
17	XP5	Shrimp	-	-	-	-	-	-	+	-	1
18	XP6	Shrimp	-	-	-	-	-	-	+	-	1
19	1P	Shrimp	-	-	-	-	-	-	+	-	1
20	2P	Shrimp	-	-	-	-	-	-	+	-	1
21	3P	Shrimp	-	-	-	-	-	-	+	-	1
22	4P	Shrimp water waste	-	-	-	-	-	-	+	-	1
23	5P	Shrimp	-	-	-	-	-	-	+	-	1

24	6P	Shrimp	-	-	-	-	-	-	+	-	1
25	7P	Shrimp water waste	-	-	-	-	-	-	+	-	1
26	8P	Shrimp water waste	-	-	-	-	-	-	+	-	1
27	DH2.1	Shrimp water waste	-	-	-	-	-	-	+	-	1
28	DH3.1	Shrimp water waste	-	-	-	-	-	-	+	-	1
No. of bacteria infected by phages			0	0	0	2	0	2	26	0	

Note: (+) – clear plaque; (-) – no plaque formation.

Plaque formation

The plaque formation ability of bacteriophage strains ΦXP4.1, ΦXP2.1 and ΦXP2.2 on the *Pseudomonas* sp. VCST7 lawn was evaluated using the double-layer agar method (Figure 3). All three phages formed clear plaques, indicating their lytic activity against the host bacteria. The plaques ranged in size from 1 to 2 mm and were surrounded by a halo, characteristic of the bullseye morphology often associated with highly lytic phages (Jurczak-Kurek *et al.*, 2016). The formation of clear plaques with a halo suggests efficient bacterial lysis and rapid propagation of the phages, supporting their potential as effective biocontrol agents against *Pseudomonas* spp., especially in the context of increasing antibiotic resistance.

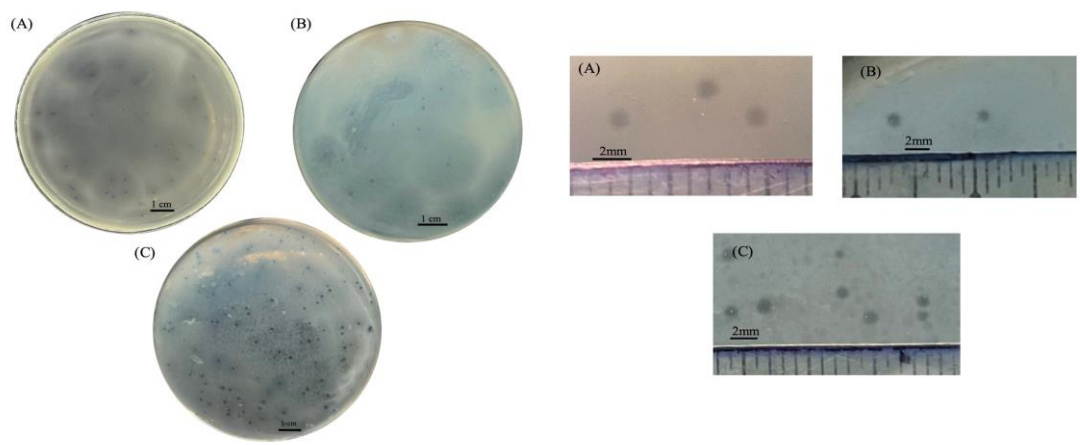

Figure 3. Plaque morphology and size of bacteriophages on (A) ΦXP4.1, (B) ΦXP2.1, and (C) ΦXP2.2 on the VCST7 bacterial lawn using the double-layer agar method.

Investigation of bacteriophage infection ability through colony density

The bactericidal activity of bacteriophages ΦXP4.1, ΦXP2.1, and ΦXP2.2 against *Pseudomonas* sp. VCST7 was further investigated using the spread plate method. The initial bacterial density of the control culture (VCST7 without phage treatment) in King's B medium was 7.814 Log10CFU/mL (Figures 4 and 5). After 24 hours of incubation, the addition of bacteriophages resulted in a reduction in bacterial density compared to the control. Bacteriophage ΦXP4.1 exhibited the most pronounced reduction in bacterial density, decreasing to

6.996 Log10PFU/mL, corresponding to an 11.08% reduction. Bacteriophages ΦXP2.1 and ΦXP2.2 also reduced bacterial density (to 7.033 and 7.025 Log10PFU/mL, respectively), but there was no significant difference between their effects. These results are consistent with the observed lytic activity of the phages, as indicated by the formation of clear plaques, and further support their potential to control *Pseudomonas* spp. growth (Sada & Tessema, 2024).

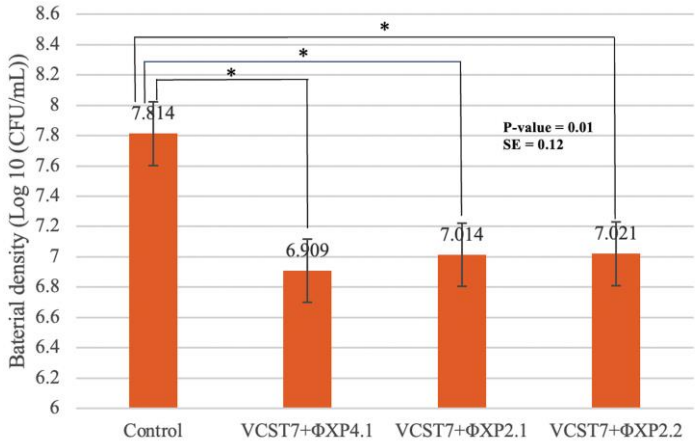

Figure 4. VCST7 bacterial density after 24-hour incubation with phages. Statistically significant differences between control values and phage treatment values are denoted by asterisks (*, $P < 0.05$, determined using Tukey's multiple-comparison test). Bars indicate the standard errors of the means.

Similar findings have been reported in other studies. For example, Van and Lieu (2024) demonstrated the lytic efficacy of bacteriophages ΦPCK1, ΦPVtH, ΦPVCt3, ΦPVCt4, ΦPDT4, and ΦPDT3 against *P. aeruginosa*, with bacteriophage ΦPCK1 reducing bacterial density by 22.03%. Adnan *et al*. (2020) showed that bacteriophage ΦMA-1 reduced the bacterial count of *P. aeruginosa*-2949.

The observed reduction in bacterial density upon phage treatment was statistically significant compared to the control ($p < 0.05$), as determined by ANOVA and Tukey's HSD post-hoc test. In conclusion, the three bacteriophages (ΦXP4.1, ΦXP2.1, and ΦXP2.2) exhibited a significant impact on *Pseudomonas* spp. strains isolated from shrimp samples, suggesting that the observed effects were not due to random chance.

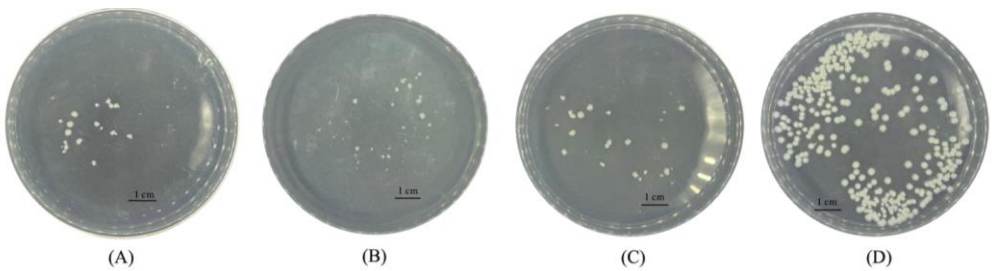

Figure 5. VCST7 colony counting plates. (A) VCST7 + ΦXP4.1, (B) VCST7 + ΦXP2.1, (C) VCST7 + ΦXP2.2 and (D) Control VCST7.

In vitro biofilm degradation assay

The ability of *Pseudomonas* sp. VCST7 to form biofilms was assessed using the crystal violet staining method after 24 and 48 hours of incubation. VCST7 was found to be capable of biofilm formation, although the extent of biofilm formation was relatively moderate with OD_{590nm} values of 0.499 and 0.600 after 24 and 48 hours, respectively. These results suggest that VCST7 to some exhibits weaker biofilm formation compared to other *Pseudomonas* strains. For example, Latz *et al*. (2017) reported *P. aeruginosa* strains PA3 and PA5 exhibiting strong biofilm formation with OD_{590nm} values exceeding 2.50.

Following treatment with bacteriophages ΦXP4.1, ΦXP2.1, and ΦXP2.2 for 4 hours, a reduction in the absorbance of crystal violet was observed, indicating a decrease in biofilm biomass (Figure 6).

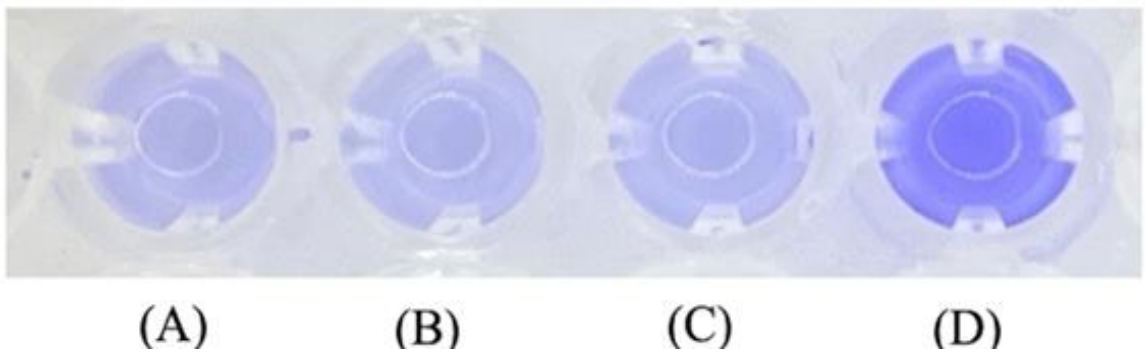

Figure 6. Biofilm staining results on a 96-well plate after 24 hours of incubation. (A) VCST7 + ΦXP4.1, (B) VCST7 + ΦXP2.1, (C) VCST7 + ΦXP2.2, and (D) Control VCST7.

Bacteriophage ΦXP4.1 demonstrated the highest biofilm reduction efficiency, decreasing the biofilm biomass from an OD_{590nm} of 0.499 to 0.168, corresponding to a 66.03% reduction after 24 hours of biofilm formation and 4 hours of phage treatment. Bacteriophages ΦXP2.1 and ΦXP2.2 also reduced biofilm formation, with OD_{590nm} values of 0.266 and 0.212 values of 0.266 and 0.212, corresponding to inhibition rates of 46.8% and 57.6%, respectively (Figure 7). These findings suggest that the tested bacteriophages can effectively inhibit biofilm formation within a relatively short treatment time (4 hours), with a statistically significant difference compared to the untreated control ($p < 0.05$).

When biofilms were allowed to develop for 48 hours before phage treatment, the biofilm reduction efficiency decreased. The control treatment had an OD_{590nm} of 0.600, while phage ΦXP4.1 reduced the OD_{590nm} to 0.452 (24.6% reduction), phage ΦXP2.1 reduced it to 0.435 (27.5% reduction), and phage ΦXP2.2 reduced it to 0.568 (5.4% reduction). This observation is consistent with the findings of Latz *et al*. (2017), who reported that bacteriophages were more effective at reducing biofilm formed by antibiotic-resistant *P. aeruginosa* strains when applied to 24-hour biofilms compared to older biofilms.

Morphological characteristics of phage *ΦXP4.1*

Based on its broad host range, significant reduction of bacterial density, and effective

biofilm degradation, bacteriophage ΦXP4.1 was selected for morphological characterization using TEM.

TEM imaging revealed that bacteriophage ΦXP4.1 possesses an icosahedral head and a distinct tail (Figure 8). The average head diameter was measured to be 61.301 nm (ranging from 56.972 to 64.521 nm, N = 10), and the average tail length was 135.7 nm (ranging from 131.06 to 139.43 nm, N = 10). Based on its morphology, bacteriophage ΦXP4.1 was classified as belonging to the class *Caudoviricetes*, which is characteristic of tailed bacteriophages, according to the classification guidelines of the International Committee on Taxonomy of Viruses (ICTV) (de Melo *et al*., 2019; Turner *et al*., 2023).

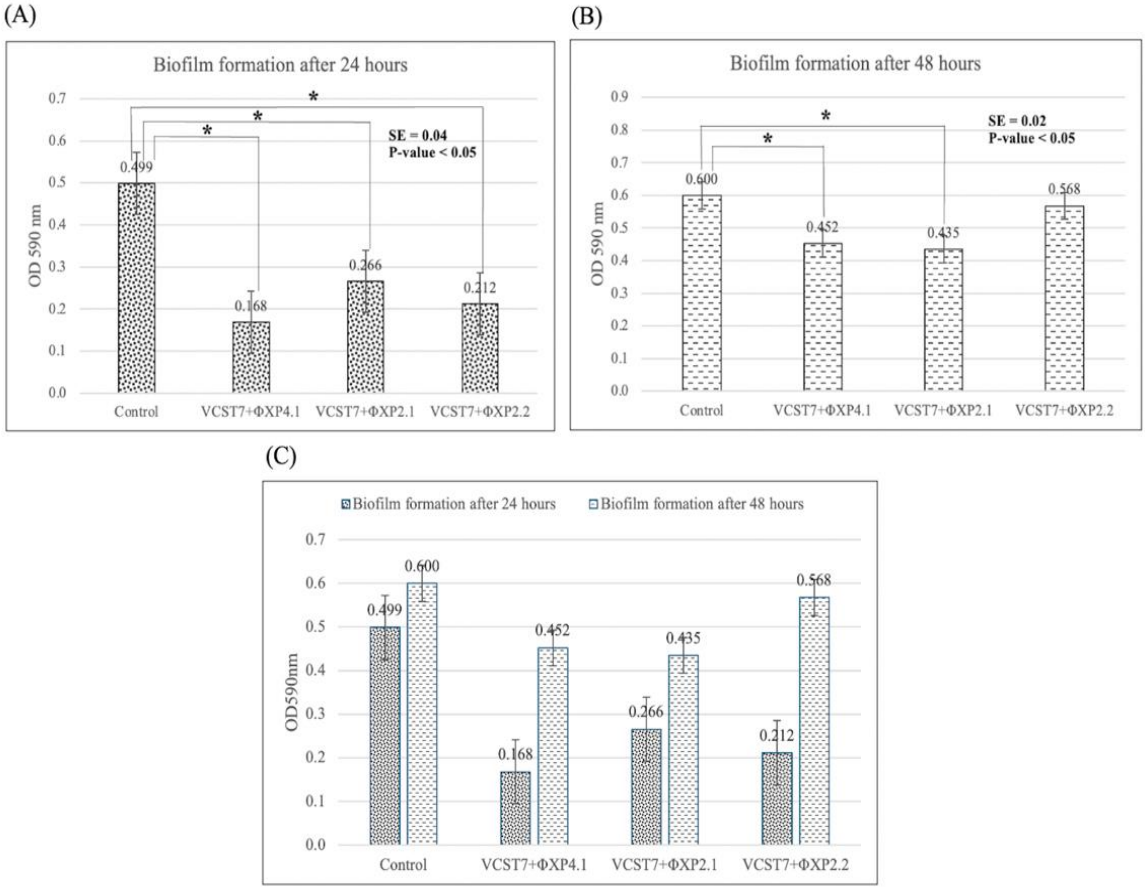

Figure 7. Effect of bacteriophages on biofilm reduction of strain VCST7. (A) After 24 hours of incubation, (B) After 48 hours of incubation, and (C) combined data from both time time points. The efficacy of the phages was evaluated based on biofilm formation measured 4 h after infection. Statistically significant differences between control values and phage treatment values are denoted by asterisk (*, P < 0.05, determined using Tukey's multiple-comparison test). Bars indicate the standard errors of the mean.

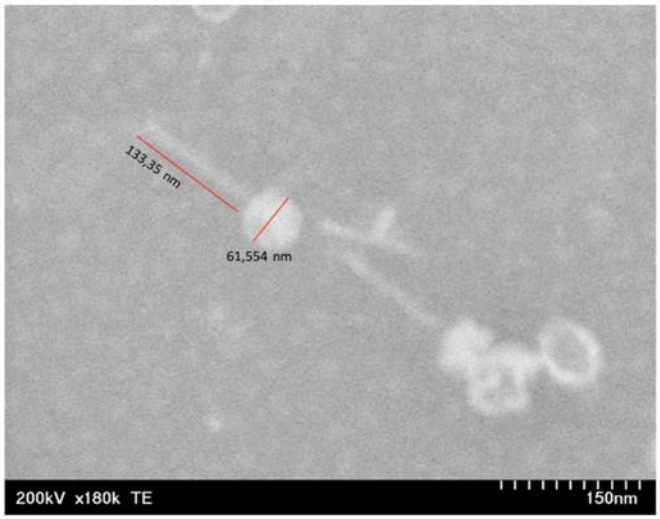

Figure 8. Morphological characteristics of bacteriophage ΦXP4.1 under Transmission Electron Microscopy (TEM) at 180,000× magnification.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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